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Transformation efficiency by Herpesvirus saimiri is not a limiting factor in clonal CD8^{pos} T cell outgrowth

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ABSTRACT

The routine transformation of human CD8^{pos} T cells by Herpesvirus saimiri has so far not been achieved in the case of pre-expanded antigen-specific CTLs. Here we transformed 73% of polyclonal EBV-specific CD8^{pos} T cell cultures using an optimized culture medium supplemented with IL-2, IL-7, IL-12, and TGF-β_1. Still, antigen-specific cytotoxicity was frequently lost and analysis of the TCR Vβ-chain repertoire revealed a variable outgrowth of several initially subdominant populations. Limiting dilution cloning of cells in the presence of high titers of HVS did not result in clonal transformation but in the rapid loss of the viral genome in outgrowing clones. In summary, our data suggest that transformation of CD8^{pos} T cells out of bulk cultures can be routinely achieved, while viral transformation itself remains an infrequent event on a per cell basis. The practical use of the improved immortalization of antigen-expanded CD8^{pos} T cell lines, however, is limited by the arbitrary outgrowth of subdominant populations of unpredictable specificity.

Introduction

The ability to virally transform antigen-specific post-thymic T cells would speed antigen identification and potentially have a role in strategies based on the adoptive transfer of lymphocytes. It is therefore a long-standing goal of workers in the field of immunotherapy. Human T cells can be transformed *in vitro* to stable growth after infection with Herpesvirus saimiri (HVS) subgroup C strain C488 (reviewed by Ensser and Fleckenstein, 2005). HVS transformed human T cell lines harbor multiple latent copies of intact, non-integrated episomal viral genomes, have a normal karyotype, and retain the expression of the T cell receptor (TCR) complex. Similarly, their functional phenotype has been shown to remain stable for months in culture, which is in contrast to T cell immortalization by HTLV-1 or by the hybridoma technique. Transformation by HVS thus allows antigen-independent, but IL-2 dependent expansion of CD4^{pos} and CD8^{pos} T lymphocytes. Following adoptive transfer into macaque monkeys, such cells even persisted for prolonged time but did not lead to T cell tumorigenesis (Knappe et al., 2000).

So far, efficient transformation by HVS has been achieved for CD4^{pos} and CD8^{pos} T cells of undetermined TCR specificity (Biesinger et al., 1992) and for antigen-specific T cell clones of the CD4^{pos} subset (Weber et al., 1993; De Carli et al., 1993; Bröker et al., 1993), but not for pre-expanded antigen-specific CD8^{pos} cytotoxic T cells (CTLs). In the single study reporting HVS transformation of EBV-specific CTLs, successful transformation of the CD8^{pos} T cells was achieved in only 1 out of 10 attempts (Berend et al., 1993). Because we have previously optimized the culture conditions for polyclonal expansion of EBV-specific CTLs under FCS free conditions, we asked whether our optimized culture system would allow a reproducible HVS transformation of antigen-specific CD8^{pos} CTLs in a higher proportion of cells.

Results

Enhanced HVS transformation of pre-expanded EBV-specific CD8^{pos} CTLs

As previously published, a RPMI 1640 based medium, termed "medium A" for the present investigation, had been optimized by our group for the transformation (Ensser and Fleckenstein, 2004). We now compared this medium A with a new serum-free, X-Vivo 15 based medium supplemented with the cytokines IL-2, IL-7, IL-12 and low dose TGF-β_1, termed "medium B". This formula was chosen under the aspect of supporting the expansion of EBV-specific CTLs. It became evident that this cytokine supplemented medium B not only

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Table 1
Efficient transformation of polyclonal CTL lines by infection with HVS in improved medium.

Exp.	Donor	Medium A			Medium B		
		Transformation	Specific lysis before transformation	Specific lysis after transformation	Transformation	Specific lysis before transformation	Specific lysis after transformation
1	A	−	+	n.t.	+	+	±
2	G	−	−	n.t.	+	−	−
3	H	−	−	n.t.	+	−	−
4	E	−	+	n.t.	+	+	+
5	A	−	+	n.t.	−	+	n.t.
6	C	−	+	n.t.	−	+	n.t.
7	A				+	±	±
8	C				+	+	−
9	F				+	+	−
10	B				+	+	−
11	D				−	+	n.t.
12	E	+	+	+			
13	G	+	+	+			
14	C	−	+	n.t.			
Parallel exp. (1–6)		0 ($n=6$)			4 ($n=6$)	0.014 p Value (Chi^2)	
All exp. (1–14)		2 ($n=9$)			8 ($n=11$)	0.025 p Value (Chi^2)	

EBV-specific polyclonal CTL lines were generated and infected with HVS viruses as described. The cultures were considered transformed, when the cultures persistently proliferated in a restimulation-independent manner 3 months after incubation with HVS. The table gives an overview about the successful transformation experiments and about maintenance of specific lytic activity ("+" = clear specific lysis, "±" = faint specific lysis, "−" = no specific lysis, n.t. = not transformed, therefore not tested). $P=0.025$ for superiority of medium B (8/11) versus A (2/9) when a Chi-Square test is applied without Yates correction; $P=0.07$ when calculated by Fisher's exact test. Six experiments (numbered 1–6) were performed with both media in parallel, from the same respective donor; for this subset of data $P=0.014$ for superiority of medium B (4/6) versus A (0/6) when a Chi-Square test is applied without Yates correction; $P=0.06$ when calculated by Fisher's exact test.

supported the growth of EBV-specific CTLs but apparently also transformation by HVS, as we obtained 8 transformations of polyclonal $CD8^{pos}$ T cells in 11 experiments. Medium A, however, supported outgrowth of transformed T cells in only two out of nine experiments (Table 1), resembling roughly the transformation frequency previously published (Berend et al., 1993). A statistical evaluation by a simple Chi-Square test indicates a $P=0.025$ for superiority of improved medium B, calculated from the total

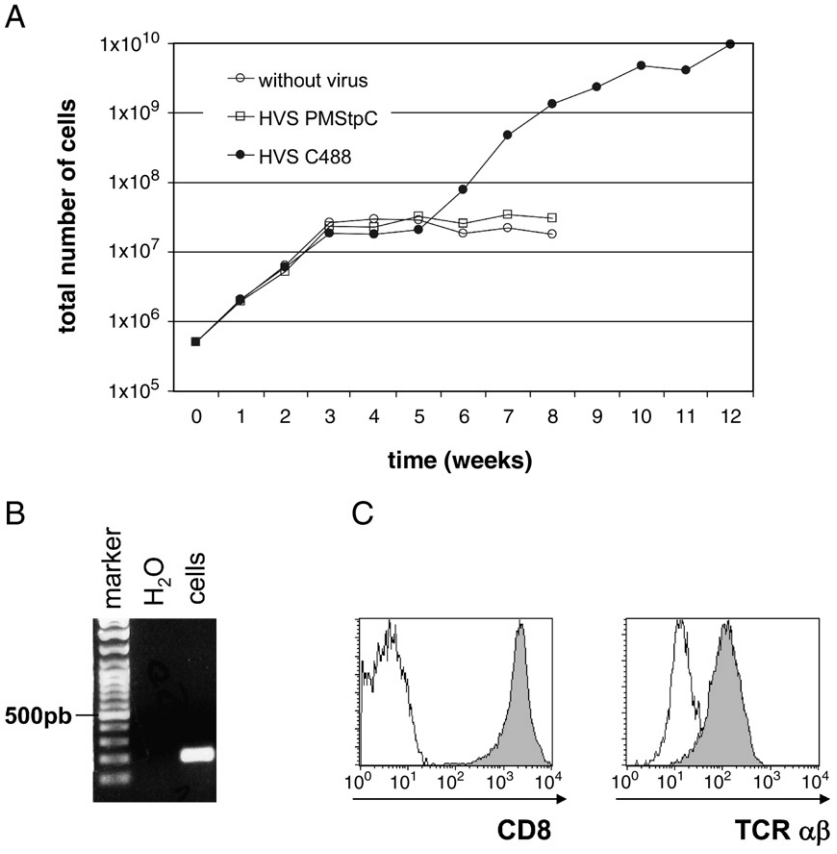

Fig. 1. Growth curve and phenotype of CTLs of a typical transformation experiment in "medium B". (A) Cell growth starting from the day of incubation with HVS, i.e. 1 day after the last restimulation with LCLs, was monitored by weekly determination of cell numbers and was calculated by taking into account the cell splitting ratio. Control cultures (in this case the culture without virus, or with the nontransforming mutant HVS PMStpC) regularly ceased from proliferation and died approximately after 8 weeks of culture. (B) Agarose gel electrophoresis showing the PCR amplification of a 194 bp DNA fragment of ORF73 from transformed cells 3 months after incubation with HVS. A 100 bp DNA ladder was chosen as a molecular weight marker. (C) The histograms show the expression of CD8 and TCR alpha-beta after 3 months of continuous culture (filled grey histograms). Open histograms with thin line represent the appropriate isotype controls.

number of successful transformation experiments 8 ($n=11$) versus 2 ($n=9$) with medium A. Calculation by Fisher's exact test indicates a $P=0.07$ for this hypothesis. Six experiments (numbered 1–6) were performed with both media in parallel, from the same respective donor; for this subset of data $P=0.014$ for superiority of medium B (4/6) versus A (0/6) when a Chi-Square test is applied without Yates correction; $P=0.06$ when calculated by Fisher's exact test. A typical cell growth curve of a transformation experiment is shown in Fig. 1A. Control cultures incubated with a non-transforming control virus (HVS PMStpC) or without virus died in parallel. Outgrowing cultures contained the HVS genome and were pure TCR-$\alpha\beta^{pos}$CD8^{pos} T cells (6 out of 6 tested cultures, Figs. 1B and C and data not shown).

Loss of EBV-specific lytic activity and outgrow of subdominant CTL clones during transformation

The single transformation of CTLs reported by Berend et al. (1993) had already shown that antigen-specific cytotoxicity can be maintained after transformation. We successfully transformed eight cultures initially possessing EBV-specific cytotoxicity and observed maintenance of function in five cultures but loss of specificity in the other three cases (Table 1). Fig. 2 illustrates examples of cultures maintaining specific lysis (donor G), acquiring of non-specific lysis (donor E), or losing specific lysis (donor F) during transformation. Since we assumed that EBV-specific polyclonal cultures initially contained also a significant fraction of non-specific bystander cells, we suspected that HVS might have mediated outgrowth of such non-specific cells in those cases, in which specific cytotoxicity was lost during transformation. By analysis of the TCR Vβ-chain repertoire we indeed detected dramatic shifts in the clonal distribution during the transformation process in that the initially broad spectrum was regularly narrowed and shifted to previously subdominant Vβ-families (Fig. 3).

Inefficient transformation of CTLs on a per cell basis

Delayed outgrowth and the expansion of only few individual clones from CTL bulk cultures indicated that transformation could be a relatively rare event on a per cell basis. We therefore tried to directly transform previously cloned EBV specific CD8^{pos} T cell lines; however, in a limited set of experiments, this approach was not successful (not shown). Alternatively, to avoid prolonged culture time before transformation (see also Discussion below) and to get a direct estimate on the transformation frequency on a per cell basis we performed limiting dilution cloning of cells and simultaneously infected them with high titers of HVS. However, serial PCR analysis of 34 outgrowing clones revealed the rapid loss of the viral genome before all (nontransformed) cultures finally stopped expansion and died (Table 2). On day 46 only the non-transforming control virus, which had been added at the highest multiplicity of infection (MOI), still gave weak signals in 8 out of 11 clones, likely reflecting residual intact viral DNA contaminating the cell pellet despite vigorous washing. These data point to a relatively low transformation frequency of human CD8^{pos} T cells by HVS also under optimized culture conditions.

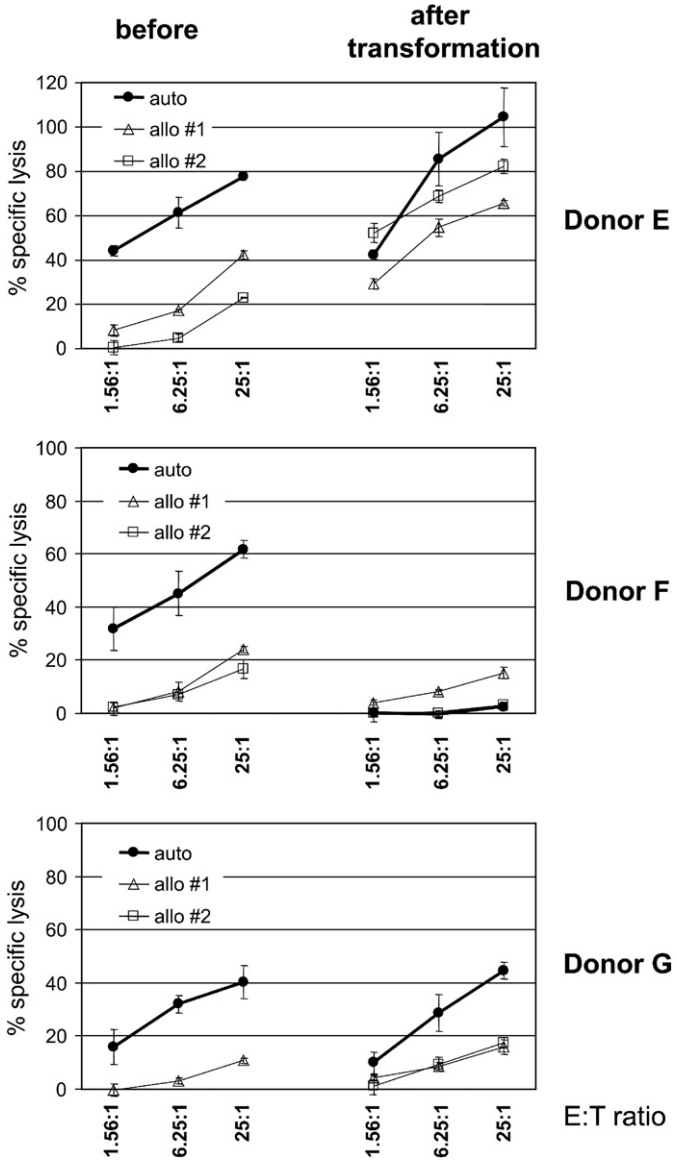

Fig. 2. Analysis of the cytolytic activity of CTLs before and after transformation. The cytolytic activity was determined on the day before and 3 months after incubation with HVS. Shown is the specific lysis of the stimulating autologous LCLs (auto) and two respective HLA non-matched allogeneic control LCLs (allo #1 and allo #2) at various effector:target (E:T) ratios expressed as mean ± S.D. ($n=3$).

Discussion

In this paper we report the improved generation of HVS transformed CD8^{pos} T cell lines from pre-expanded polyclonal antigen specific bulk cultures. We achieved this by using a serum-free medium previously optimized for the generation of EBV-specific CTLs by adding a low concentration (1%) of human serum together with IL-2, IL-7, IL-12, and low dose TGF-β_1 (Holter W., unpublished data). Although TGF-β_1 has been widely implicated in the generation and also the effector function of regulatory T cell populations in mice, it does not necessarily induce a regulatory phenotype in human T cells (Tran et al., 2007), and has been found previously by us to rescue T cells from activation induced cell death and to support a T cell effector phenotype characterized by high IL-2 production (Cerwenka et al., 1994; Cerwenka et al., 1996). IL-7 was chosen for its known importance in maintaining memory T cells (Schluns and Lefrancois, 2003) and IL-12, since this cytokine might support clonal expansion of cytolytic and helper T cells (Felzmann et al., 2005; Trinchieri, 2003). Under these improved culture conditions we achieved transformation of over 70% of bulk cultures, comparing favorably with the previously reported transformation of 1 out of 10 experiments (Berend et al., 1993). The outgrowth of transformed cells appeared rather late, when extensive cell death was already observed in parallel cultures maintained in the presence of only IL-2. These data suggested to us that the improved survival of T cells generated in the presence of a low dose of TGF-β_1, along with IL-7 and IL-12, was helpful for HVS transformation of the cells.

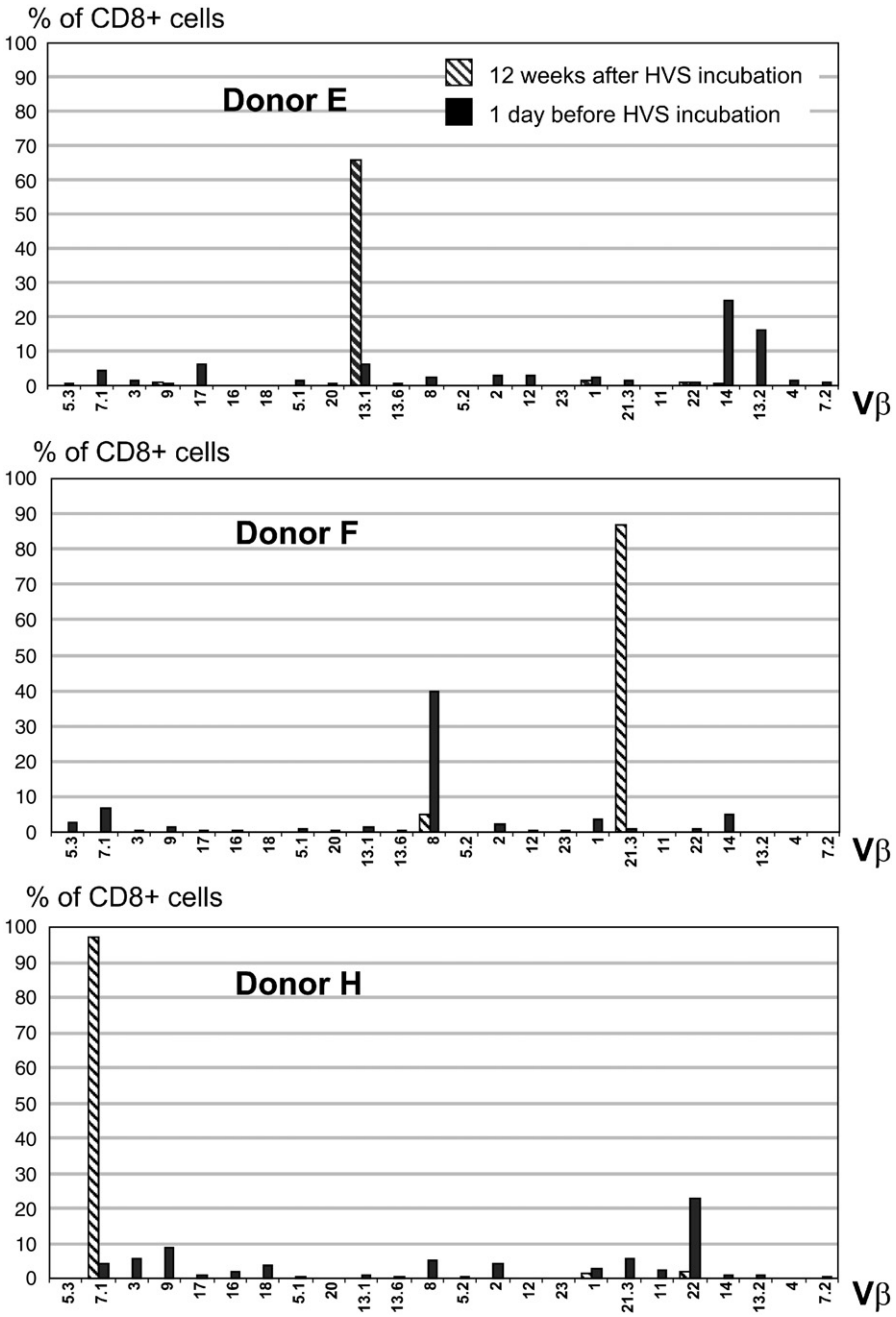

Fig. 3. Analysis of the TCR Vβ-chain repertoire before and after transformation. Flow cytometric analysis of the polyclonal $CD8^{pos}$ CTLs, after 4 weekly stimulations on the day before and 3 months after incubation with HVS, exemplified by three different experiments.

Even under optimized culture conditions, however, transformation turned out to be still inefficient on a per cell basis. This is indicated by experiments in which limiting dilution cloning was performed immediately after infection with HVS. Here we observed that the viral genome progressively disappeared in all outgrowing clones. Loss of viral DNA due to overgrowth of non-infected cells or due to induction of anti-HVS-specificity can be excluded under these conditions, as the clones arose from single cells. Our impression of a relatively inefficient transformation on a per cell basis is in line with the fact that a minimum number of several million cells has originally been recommended for efficient transformation (Biesinger et al., 1992) and a frequency of about 1 in 10^5 T cells was reported at lower MOI for cord blood lymphocytes (Fickenscher et al., 1997).

Table 2
Progressive loss of viral DNA in growing cell clones after limiting dilution.

	MOI (for 6 h)	15 days after limiting dilution	26 days after limiting dilution	46 days after limiting dilution
HVS C488	132	5/8	7/34	0/11
HVS Mix121	1280	7/7	19/19	8 (weak)/11

The table shows the number of outgrowing clones and the number of clones tested positive for the HVS genome (given as HVS genomic DNA positive clones/outgrowing clones) after different time after limiting dilution of the freshly infected EBV-specific polyclonal CTL lines.

In addition, our data show that HVS transformed CTL cultures regularly lose their polyclonal character. This could be expected assuming clonal overgrowth during prolonged proliferation and would also be in line with a low fraction of cells being transformed. Unexpectedly, however, the TCR-Vβ family phenotyping showed that

the clones growing out during the long process of transformation had been initially of low frequency only. This fact could possibly be explained by replicative senescence in the initially most strongly expanded $CD8^{pos}$ clones, thus hindering the process of transformation. In general, the activation and stimulation of T cells is associated with an induction in TERT activity, and replicative senescence and immune exhaustion has been associated with Telomere shortening (reviewed by Akbar and Vukmanovic-Stejic, 2007). In this context, it is important that $CD8^{pos}$ clones, in contrast to $CD4^{pos}$ clones early lose telomerase activity (reviewed by Effros, 2004). Transformation by HVS has been shown to increase telomerase reverse transcriptase (TERT) activity in $CD4^{pos}$ T cells by Harnack et al. (2001). We have measured the relative telomere length by the flow cytometry PNA/FISH technique in CD8 cells from three donors before and after transformation in a pilot experiment. This revealed an apparent increase in relative telomere length after HVS transformation in all three cell lines (data not shown). The inability to expand CD8-positive clones by HVS is probably not caused by telomere exhaustion, as this might be overcome by HVS by an as yet unknown mechanism. The observed longer telomeres after HVS transformation and its mechanism may significantly contribute HVS transformation, and will be studied in a separate research project.

Alternatively, it has been recognized that only minimal differences in kinetic parameters of individual cells can rapidly lead to large differences in clonal growth (Saadawi et al., 1997). Both possible explanations have negative consequences for the maintenance of a desired antigen-specificity when transformation is performed with polyclonal cultures containing a considerable fraction of bystander cells with unknown reactivity. These limitations of HVS transformation should therefore be kept in mind when considering the technique to generate $CD8^{pos}$ transformed T cell lines of any desired specificity.

Materials and methods

Generation of EBV-specific T cell lines and HVS transformation

Five times 10^6 human mononuclear cells (MNCs) were stimulated with 1×10^6 autologous irradiated (60 Gy) EBV-transformed B-cell lines (LCLs) in 10 ml of X-Vivo 15 supplemented with 1% HS. IL-2 was added at 1 U/ml on day 0 and day 7, at 10 U/ml on days 3, 10, 14 and 21 and at 100 U/ml on day 17 and day 24. 10 U/ml IL-7 was added beginning on day 7. Restimulation with irradiated LCLs was done on day 7 at a responder/stimulator ratio of 5:1 and on day 14 and day 21 at a ratio of 1:1. FACS analysis and functional phenotyping was performed on day 21 or day 28 of culture. The TCR Vβ repertoire was analyzed by IOTest® Beta Mark TCR Vβ Repertoire Kit covering about 70% of the human TCR Vβ repertoire (Beckman Coulter) and the cytotoxicity was determined by a Europium based assay as previously described (Lehner et al., 2007). For transformation experiments 200 µl of supernatants containing the wild type HVS C488, or the non-transforming HVS mutants Mix121 or PMStpC (deletions in the stpC oncogene) were added to 2×10^6 cells of the polyclonal cultures 1 day after restimulation in 2 ml of medium A (RPMI-1640 supplemented with 50% Panserin 401; Pan Biotech, Aidenbach, Germany) or medium B (X-Vivo 15 supplemented with 1% HS, 10 U/ml IL-2, 10 U/ml IL-7, 1 U/ml IL-12 and 1 ng/ml TGF-β_1). The cultures were fed twice a week.

Limiting dilution cloning and detection of the HVS genome by PCR

Transformation efficiency at the single cell level was determined by infection of an aliquot of the polyclonal CTL bulk cultures on day 21. During infection 240 cells were incubated in 200 µl of medium B containing 1.7×10^5–2.5×10^7 HVS virions/ml. After 6 h the cells were diluted 120-fold without washing the virus away. One cell per well in 100 µl medium B containing 100 U/ml IL-2 was seeded in a round bottom 96-well plate. 10,000 irradiated autologous LCLs were added on day 0 and day 7 after seeding. The cultures were fed twice a week with cytokine containing medium B. For PCR analysis the cells were washed thrice to remove residual intact virus particles present in the culture medium. After the last centrifugation each supernatant was kept as a control. Washed cell pellets and supernatants were digested with proteinase K and subjected to PCR amplification of the ORF73 of the HVS genome or of GAPDH. Primers for HVS-ORF73: 5′-GTGCTACTCACATTGAAAATCGAAACTTC-3′ and 5′-GGTTTAACATATGTTTTGCGGTTGC-3′; human GAPDH: 5′-GCAGGGGG-GAGCCAAAAGG-3′ and 5′-TGCCAGCCCCAGCGTCAAAG-3′.

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